Identification and Characterization of Compounds with Inhibitory Properties towards *Salmonella enterica* Biofilms

Undergraduate Thesis

Presented in partial fulfillment of the requirements for graduation with research distinction in Microbiology in the College of Arts and Sciences at The Ohio State University.

Jasmine S. Moshiri

Bachelors of Science in Microbiology
College of Arts and Sciences
The Ohio State University
2016

Thesis Committee:
John Gunn, Ph.D., Advisor
Natividad Ruiz, Ph.D.

Table of Contents

Acknowledgements

I would like to sincerely acknowledge the tireless support and guidance provided by Dr. John Gunn, who I have had the privilege of working with throughout my undergraduate career. Thank you, Dr. Gunn, for the time and energy you invest in training undergraduate students; I feel extremely fortunate to have benefited from the mentorship of a faculty member who prioritizes training young scientists as you do. I would also like to acknowledge Jacob Koopman and Joanna Marshall, who served as excellent mentors when I joined the lab and provided valuable training. Additionally, I would like to thank past and present laboratory members including Ky Hoang, Juan Gonzalez, Haley Adcox, Erin Vasicek, Dominique Olds, Olivia Harder, Darpan Kaur, Michael Neiger, and Brad Eichar for their support. Thank you to our collaborators Chido Hambira, Jim Fuchs, Santosh Salunke, and Ching-Shih Chen in the Ohio State University Department of Medicinal Chemistry and Pharmacognosy. I would like to thank Dr. Natacha Ruiz for her support as well as for her service on my thesis committee.

Lastly, I would like to acknowledge the funding sources for this work, which include the National Institutes of Health, the SOLAR Research Fund, the Undergraduate Education Summer Research Fellowship Program, OSU Biofilms in Human Medicine Group, OSU Public Health Preparedness for Infectious Disease, and the OSU Dean's Discovery Program.

Chapter 1

Introduction

1.1 Problem Statement

Salmonella enterica (*S. enterica*) is a facultative, intracellular, gram-negative bacterium that causes serovar-specific disease in a range of hosts. Over 2,500 serovars of *S. enterica* have been identified based on flagellar and lipopolysaccharide (LPS) antigen specificity [1]. In the human host, *S. enterica* serovars may instigate typhoidal as well as non-typhoidal illness, both with significant disease prevalence worldwide. Non-typhoidal *S. enterica* is responsible for over 78 million cases of foodborne illnesses and over 59,000 deaths globally each year [2]. *Salmonella enterica* serovar Typhi (*S.* Typhi), the causative agent of Typhoid Fever, infects and kills an estimated 20 million and 200,000 individuals each year, respectively [3]. *S.* Typhi is of high clinical relevance in the regions of Southern Africa, South-central Asia, and South-eastern Asia, where contamination of both food and water sources in developing nations allows *Salmonella* to spread to new hosts [3].

Upon ingestion of *S.* Typhi, successful bacteria will persist through the gastric barrier of the stomach to reach the small intestine. At this location, *S.* Typhi crosses the intestinal epithelium and is phagocytosed by macrophages to achieve systemic disease [4]. Sites commonly infected during systemic salmonellosis include the ileum, liver, spleen, bone marrow, and gallbladder [5].

After resolution of the acute infection, *S.* Typhi can persist in the gallbladder of 3-5% of hosts through an asymptomatic chronic carrier state of disease [6]. During chronic carriage, *S.* Typhi forms biofilms—communities of microorganisms that adhere to

surfaces and are recalcitrant to immune and antibiotic clearance—on the surface of gallstones and the gallbladder epithelium [5, 7]. The process of biofilm formation is characterized by sequential events that include initial attachment to a surface, secretion of a protective extracellular polymeric substance (EPS), and dispersal of community members to the environment [8] (Figure 1). As a human-restricted pathogen, the spread of *S.* Typhi is dependent on this carrier state during which fecal shredding allows bacteria to spread to new hosts through contamination of water sources. Thus, the study of *S. enterica* biofilm processes is fundamental to impeding the spread of *S.* Typhi via chronic carriage.

1.2 Hypothesis and Rationale

The central hypothesis for this thesis is that we can identify and characterize compounds with the ability to disrupt the process of *S. enterica* biofilm formation. Utilizing a model organism, *Salmonella enterica* subspecies enterica serovar Typhimurium (*S.* Typhimurium), this hypothesis was examined with the three following aims:

1. Identification of compounds with anti-biofilm properties.
2. Examination of the characteristics and activities of identified anti-biofilm compounds.
3. Evaluation of the mechanism of action of anti-biofilm compound T315.

The discovery and characterization of compounds with the ability to specifically inhibit early events in bacterial attachment will aid future studies of *Salmonella* biofilm

formation and is a promising step towards eradication of the *Salmonella* chronic carriage state.

Chapter 2

Materials and Methods

2.1 Bacterial strains, growth conditions, and kinase inhibitors

The bacterial strains used in this study were *S.* Typhimurium 14028s (JSG210), *Acinetobacter baumannii* (JSG3828) and *Pseudomonas aeruginosa* PAO1 (JSG3906). Overnight cultures were grown in LB broth at 37°C with aeration. A 24h rapid attachment or temporal addition assay for JSG210 included normalizing to OD_{600nm}=0.8, diluting 1:100 into 1:20 TSB, and incubating 24h post drug addition at 30°C in static conditions. 24h rapid attachment assays for JSG3828 and JSG3906 included normalizing to OD_{600nm}=0.8, diluting 1:100 into LB, and incubating 24h post drug addition at 30°C in static conditions. Kinase inhibitor stocks were stored in DMSO at 5 mM. Unless otherwise indicated, drugs were administered via a 1:10 dilution of a 50 μM working solution of 1% DMSO in PBS, yielding a final drug concentration of 5 μM.

2.2 Rapid Attachment and Temporal Addition Assays

Biofilm Rapid Attachment Assays were conducted using growth conditions and drug concentrations described above with drug added prior to incubation in static conditions. 24h post-drug addition, growth was read at OD_{600} and plates were washed 4 times in ddH_20 to eliminate planktonic cells. Biofilms were heat fixed 1h at 60°C, stained with 33% crystal violet solution (6.0 mL PBS, 3.3 mL crystal violet, 333 μL methanol, 333 μL isopropanol) for 5 min, and released using 33% acetic acid (3.3 mL glacial acetic acid, 6.7 mL ddH_20) to quantify the stain. Biofilm quantification was performed by reading the released dye at OD_{570}. Temporal Addition Assays were conducted using the

same conditions and crystal violet technique; however, drug addition was delayed to 1, 3, and 6h post-initial incubation, with 15 min on orbital shaker after each addition to ensure even drug dispersal.

2.3 Viability Assay

To determine bactericidal or bacteriostatic properties, *S.* Typhimurium and *A. baumannii* were grown as previously described (37°C in LB, aerobic conditions) in the presence or absence of drug at concentration of 5 or 10 μM. Samples were taken at 0, 2, 4, 6, 8, 16, and 24h after start of growth, serially diluted, and plated for enumeration.

2.4 EC_{50} Determination

The half maximal effective concentration (EC_{50}) was calculated using measurements of biofilm inhibition at concentrations of drug from 0.5 to 9 μM (24h at 30°C) via the Rapid Attachment Assay. Biofilms were quantified using the crystal violet stain and dye release technique.

2.5 Generation of T315-Resistant Mutants

Following 24h exposure of *S.* Typhimurium to 5 μM T315 at 30°C, cells residing within the biofilm were cultured in LB at 37°C and exposed to 5 μM T315 three additional times before increasing drug exposure to 7.5 μM and then 10 μM. Additional test well replicates were included from which biofilm-residing *S.* Typhimurium were not obtained, and these wells were stained with crystal violet to observe development of resistance to T315. Cultures at each stage were stored in 20% glycerol at -20°C. This

procedure was performed in triplicate. Resistance to T315 by an isolated mutant obtained from biofilms after the 10 μM exposure will be confirmed via the Rapid Attachment Assay with 10 μM T315 before submitting mutant gDNA for whole-genome sequencing.

2.6 Direct Pull-Down of T315 and Target

A polyethylene glycol (PEG) linker and biotin group will be added to T315 via click-chemistry. After confirmation that PEGylation and biotinylation of T315 does not inhibit anti-biofilm activity, biotinylated T315 will be exposed to a *S.* Typhimurium cellular lysate (obtained via sonication in 50 mM Hepes pH 7.4, 120 mM NaCl, 20 mM $MgCl_2$, 0.1% Triton X-100, and 1x commercial complete protease inhibitor cocktail) and T315-bound target will be isolated via streptavidin affinity chromatography using the Pierce™ Biotinylated Protein Interaction Pull-Down Kit. Target identification will be achieved via mass spectrometry.

Chapter 3

Results

3.1 Identification of kinase inhibitor compounds with anti-biofilm properties

An initial screen of 90 derivatives of kinase inhibitor compounds against *S.* Typhimurium was performed using 5 μM of compound for 24h at 30°C. Biofilms were assessed via crystal violet incorporation. Through this technique, two compounds were identified that showed promising anti-biofilm activity: T315 and Cp315. The structure of T315, as well as regions of T315 structurally identical to the closely related Cp315 molecule, are represented in Figure 2.

Rapid attachment assays performed in triplicate show that T315 and Cp315 at concentrations of 5 μM inhibit *S.* Typhimurium biofilm formation by 59.4% and 58.4%, respectively. Previous work with T315 indicate that it has anti-cancer properties, as it has been demonstrated to disrupt the PI3K/AKT pathway, which contributes to cancer progression [9]. Previously tested prostate and mammary epithelial cells did not show cytotoxicity with T315 at concentrations within 1-5 μM [10], and the half-maximal lethal dose of T315 against normal T cell and B cell lymphocytes was greater than 10 μM [9].

3.2 Characterization of the phenotypic effects of promising anti-biofilm agents T315 and Cp315

3.2 a. Evaluation of bacteriostatic or bactericidal effects

Viability assays were used to determine the effects of 5 μM T315 and Cp315 on planktonic growth of *S.* Typhimurium. Over a 24h time-course at 37°C, no substantial differences of *S.* Typhimurium growth was observed between T315 and Cp315 as

compared to a no drug control of 1% DMSO in PBS, as shown in Figure 3. These data indicate that neither T315 or Cp315 cause a decrease in biofilm formation as a result of bacteriostatic or bactericidal effects.

3.2 b. Characterization of temporal relationships of drug activity

To better characterize the temporal relationship of drug activity with biofilm formation, drug administration was delayed by 1, 3, and 6h post-initial incubation. Delaying drug administration allows cells to begin the adherence process before exposure to drug and differentiates between preventative and dispersal anti-biofilm effects. As shown Figure 4, delayed administration of drugs results in a reduction in their anti-biofilm activity. At drug administration 6h post-initial incubation (pii), 5 μM T315 exhibited only a 20% reduction in biofilm formation, as compared to 30% reduction at 3h pii, and 42% at 1h pii. Similarly, 5 μM Cp315 exhibited 18% reduction in biofilm formation at 6h pii, 25% reduction at 3h pii, and 49% at 1h pii. Thus, even a one-hour allowance for *Salmonella* to begin the biofilm formation processes prior to drug exposure reduced the anti-biofilm activity of both compounds as compared to activity quantified in rapid attachment assays.

3.2 c. Determination of half-maximal effective concentration (EC_{50}) of T315

In order to evaluate the efficiency of T315 activity, the half-maximal effective concentration (EC_{50}) was calculated by evaluating anti-biofilm activity at a range of concentrations between 0.5 and 9μM. Figure 5 depicts the reduction in *S.* Typhimurium biofilm observed by exposure to T315 at these varying concentrations. The EC_{50} was determined to be equal to 4.61 μM with a 95% confidence interval from 3.13 to 6.78 μM.

3.2 d. Characterization of T315 activity against additional gram-negative biofilm-forming pathogens *A. baumannii* and *P. aeruginosa*

The anti-biofilm activity of T315 towards gram-negative pathogenic biofilm forming species *A. baumannii* and *P. aeruginosa* was evaluated (Figure 6a). 24h rapid attachment assays at 30°C indicate that at a concentration of 10 μM, T315 significantly reduces *A. baumannii* (JSG3828) biofilm formation by an average of 61.2% (p-value < 0.001). At the 5 μM concentration tested, T315 was not shown to significantly affect biofilm formation of *P. aeruginosa* PAO1 (JSG3906). Enumeration of planktonic growth *A. baumannii* at timepoints over 24h at 37°C indicates that 10 μM T315, while inhibiting biofilm formation, does not work through bacteriostatic or bactericidal effects (Figure 6b).

3.2 e. Testing of T315 derivative In-T315 for anti-biofilm activity in *S.* Typhimurium

A compound closely related to T315, termed In-T315, was obtained and evaluated for anti-biofilm activity in *S.* Typhimurium. In-T315 relates to T315 in that the central portion of the molecule has been modified to include an additional aromatic ring, providing steric bulk to In-T315 while keeping side groups identical to those of T315. Assessing biofilm formation with crystal violet staining technique, *S.* Typhimurium biofilm formation 24h at 30°C, we observed that In-T315 exhibited a high level of anti-biofilm activity at concentrations of 5 and 10 μM.

Figure 7 illustrates the average biofilm formation observed when JSG210 was exposed to both Cp315 and In-T315 at a concentration of 5 μM as well as In-T315 at 10 μM. At both concentrations tested, Cp315 and InT315 significantly reduced biofilm

formation of JSG210. Cp315 exhibited an average of 58.4% inhibition at 5 μM. In-T315 exhibited an average of 74.8% biofilm inhibition at 5 μM and an average of 83.3% biofilm inhibition at 10 μM against JSG210.

3.2 f. Characterization of T315 anti-biofilm activity against *S.* Typhimurium surface mutants

T315 activity against *S.* Typhimurium surface appendage mutants was characterized in hopes of providing insight to T315 functionality. Identification of *S.* Typhimurium mutants used in these studies is included within Table 1 and includes strains with reduced expression of curli fimbriae (JSG3736), motility (JSG1547), and flagellar appendages FljB (JSG1178) and FliC (JSG1179) in a *S.* Typhimurium ATCC 14028s background. The ability of a *S.* Typhimurium SR11 quadruple fimbriae operon mutant (JSG1174) to form biofilms in the presence of T315 was also characterized. Strains were exposed to 5 and 10 μM T315 for 24h at 30°C, and biofilm-residing cells were stained with crystal violet for quantification of biofilm formation. The level of inhibition of biofilm formation observed due to the presence of T315 in the JSG3736, JSG1547, JSG1178, and JSG1179 strains is depicted in Figure 8 and can be compared to that observed in the JSG210 wild type *S.* Typhimurium 14028s strain. In these studies, JSG1178 exposed to 10 μM T315 experienced an average of 58.9% inhibition of biofilm formation while JSG210 displayed 78.4% inhibition at this concentration; this difference is statistically significant. JSG1178 is deficient in expression of the *S.* Typhimurium FljB flagellum, suggesting that this appendage may be a target of T315 compound, as the compound exhibits reduced biological activity with the absence of this structure. JSG1547, JSG3736, and JSG1179 bacterial strains also shown in Figure 8a did not

experience a statistically significant change in biofilm inhibition levels compared to the JSG210 wild type background strain at any concentration of T315.

The anti-biofilm activity of T315 against a *S.* Typhimurium SR11 quadruple fimbriae mutant (JSG1174) was studied at both 5 and 10 μM concentrations. Quantification of biofilm formation was achieved after 24h exposure at 30°C through crystal violet staining. JSG1174 is deficient in *fim, lpfC, pefC,* and *agfB* fimbriae structures, and the response of this mutant to T315 was compared to SR11 wild type strain JSG1169. No significant difference was observed of T315 anti-biofilm activity towards JSG1174 as compared to the JSG1169 wild type background (Figure 8b), suggesting that these fimbriae structures are not involved in the mechanism of action of T315.

3.3 Evaluation of the mechanism of action of T315

3.3 a. Generation of a T315-resistant *S.* Typhimurium isolate

To understand the mechanism of action of T315, an attempt was made to generate a *S.* Typhimurium JSG210 isolate that successfully formed biofilms in the presence of the compound. Whole-genome sequencing would then be performed on this isolate to identify loci altered from the wild-type and thus identify genes and gene products that T315 interacts with. Following 24h exposure of *S.* Typhimurium to 5 μM T315 at 30°C, cells residing within the biofilm were obtained and exposed to 5 μM T315 three additional times before increasing drug exposure to 7.5 μM and then 10 μM. Additional replicate wells were included which were not disturbed by the process of acquiring biofilm-residing cells, and these cells were stained with crystal violet to monitor the

development of resistance to T315. We did not observe the development of any lasting resistance in any of the three biological replicates performed. A transient resistance was seen during repetition of the 5 μM drug exposure level, but this phenotype did not persist upon an increase to 7.5 μM and subsequently 10 μM; this increase in drug concentration from 5 μM was attempted twice.

3.3 b. Direct Pull Down of T315 and Target

Attachment of a polyethylene glycol (PEG) linker to T315 was achieved through collaboration with the lab of Dr. Jim Fuchs at The Ohio State University via utilization of click-chemistry. Synthesized PEG-T315-i included attachment of the PEG linker at the site of the piperazine functional group. PEG-T315-i was evaluated for retention or reduction of anti-biofilm activity. As depicted in Figure 9b, PEG-T315-i exhibited a loss of biological activity. Given this information, we suspect that the (i) site is necessary for the biological activity of T315. The polyethylene glycol linker is currently being attached at the amide (ii) site of T315. If this location of PEG linkage retains biological activity, a biotin marker will be attached, and this molecule will be used for direct pull down of JSG210 target and mass spectrometry analysis.

Chapter 4

Discussion

The *S.* Typhi chronic carriage state includes the formation of biofilm communities on the surface of gallstones in the gall bladder and on the gall bladder epithelium. Recalcitrance of biofilms to antibiotic clearance results in difficulty resolving the chronic infection. Targeting the biofilm formation process with anti-biofilm therapeutics could be used in conjunction with antibiotic agents to eliminate the biofilm reservoir within *Salmonella* carriers. In efforts to identify compounds that counter *Salmonella* biofilm processes, we hypothesized that we can identify and characterize compounds with the ability to disrupt the process of *S. enterica* biofilm formation. This hypothesis was examined by (1) the identification of compounds with anti-biofilm properties, (2) the examination of the characteristics and activities of identified anti-biofilm compounds and (3) the evaluation of the mechanism of action of anti-biofilm compound T315.

4.1 Identification of compounds with anti-biofilm properties

Through a screen of 90 derivatives of kinase inhibitor compounds, we identified two highly related compounds, T315 and Cp315, which significantly reduced the formation of *S.* Typhimurium biofilms. At the 5 μM concentration used in the preliminary screening, T315 and Cp315 inhibited biofilm formation by 59.4% and 58.4%, respectively. T315 has previously been characterized as an anti-cancer therapeutic that targets the PI3K/AKT pathway. Studies indicate that it does not induce host cell cytotoxicity in a number of human epithelial cell lines, including prostate and mammary epithelial cells as well as T and B lymphocytes.

4.2 Examination of the characteristics and activities of identified anti-biofilm compounds

Due to the promising anti-biofilm activity of T315 and Cp315 observed in the preliminary screen, the functionality of these compounds were further characterized. First, the bacteriostatic or bactericidal activity of the compounds were evaluated by exposing *S.* Typhimurium to 5 μM of each compound and plating for enumeration at several timepoints over 24h. *Salmonella* viable cell density during planktonic growth did not appear to be affected by the presence of either T315 or Cp315 in these studies, which suggests that the decrease in biofilm formation observed is not a resultant of bacteriostatic or bactericidal activity of the compounds. Instead, the compounds interfere with a bacterial process specific to the biofilm lifestyle.

To better understand the temporal relationship between drug addition and effectiveness, drug administration was delayed 1, 3, and 6h to allow bacterial cells to begin biofilm formation prior to drug exposure. 24h post-exposure, biofilms were quantified by crystal violet staining, and we noted that delaying drug exposure impacted the effectiveness of both T315 and Cp315 at 5 μM. Delaying T315 addition 1, 3, and 6h post-addition of cells resulted in an average of 41.5%, 30.1%, and 19.5% biofilm inhibition, respectively, compared to no drug. An identical experiment with Cp315 resulted in an average of 48.5%, 25.3%, and 17.7% biofilm inhibition, respectively, compared to no drug. When added at the same time as initial incubation of *Salmonella* the same concentrations of T315 and Cp315 inhibited biofilm formation by 59.4% and 58.4%, respectively. When drug administration was delayed to 24h post-initial incubation

of cells, when formation of a mature biofilm is underway, less than 3.5% difference between biofilm formation with and without the presence of either compound was observed (data not shown). These data indicate that both T315 and Cp315 are most effective when administered early in the biofilm formation process and are ineffective at dispersing a mature biofilm. This suggests that structures involved in the early attachment stage of biofilm development, such as the expression of surface appendages, may be affected by T315 and Cp315.

Due to the structural and functional similarities we observed between T315 and Cp315, we chose to continue our characterization studies by focusing on T315. Previous studies conducted on the anti-cancer properties of T315 described the cytotoxic effects of the compound and the compound structure [9, 10]. We determined the half-maximal effective concentration of T315 anti-biofilm properties towards *S.* Typhimurium to be 4.61 μM with a 95% confidence interval from 3.13 to 6.78 μM. This value is comparable to the 5 μM drug concentration we have used throughout these studies. Issues with T315 solubility in 1x PBS, described in an upcoming section, prevented our EC_{50} studies from exceeding 10 μM, which is a limitation of this work.

The effectiveness of T315 anti-biofilm activity towards gram-negative biofilm forming pathogens *A. baumannii* and *P. aeruginosa* was characterized. 10 μM T315 is effective at reducing *A. baumannii* biofilm formation by an average of 61.2%, and does not appear to exhibit bactericidal or bacteriostatic effects on planktonic growth. *P. aeruginosa* biofilms were unaffected by T315 in these studies, which indicates that T315 is a specific biofilm inhibitor. These data suggest that *S.* Typhimurium and *A. baumannii* share one or more biofilm-formation processes which are not necessary for *P. aeruginosa*

biofilm formation, and that these processes are targeted by T315 to reduce biofilm formation in a species-specific manner.

A compound closely related to T315, In-T315, was obtained from the lab of Ching-Shih Chen for examination of potential anti-biofilm activity. In-T315 side groups are identical to those of T315, but has a modified central structure that provides additional steric bulk to the molecule. In these studies, exposure of *S.* Typhimurium to In-T315 resulted in an average of 74.8% biofilm inhibition at 5 μM and an average of 83.3% biofilm inhibition at 10 μM. These data suggest that In-T315 may be a more potent anti-biofilm agent than T315; however, due to the inherent variability of these studies as a result of compound instability in solution these results should be further replicated to confirm this finding.

Due to observations that early addition of drug to bacterial static growth is necessary for maximum anti-biofilm activity, we hypothesize that structures on the cellular surface involved in early attachment phases of biofilm formation are affected by the presence of T315. *S.* Typhimurium strains with mutations in curli fimbriae (Δ*csgA*), motility (Δ*motA*), FliC flagellum (Δ*fliC*), FljB flagellum, and fimbriae operons (Δ*fim* Δ*lpfC* Δ*pefC* Δ*agfB*) were obtained and T315-induced inhibition of biofilm formation was compared to that of appropriate background strains. A significant reduction in anti-biofilm activity of T315 was observed in the JSG1178 strain, which is deficient in expression of the FljB flagella appendage. When exposed to 10 μM T315 for 24h, JSG1178 experienced an average of 58.9% inhibition of biofilm formation compared to 78.4% inhibition of biofilm formation that the background strain JSG210 displayed at this concentration. This reduction in efficiency of drug activity suggests that the FljB

structure altered in the JSG1178 strain is involved in the functionality of T315. The role of FljB in *S.* Typhimurium biofilm formation is inconclusive, which complicates this analysis. FljB and FliC, *S.* Typhimurium flagellar filament structures, are controlled by an expression switch. Studies conducted comparing *S.* Typhimurium attachment to cholesterol-coated surfaces conclude that FliC, but not FljB, promote biofilm formation [11], while studies on polystyrene at 37°C concluded that the absence of both FliC and FljB is beneficial to biofilm formation [12]. In this work, the level of biofilm formation between JSG210 and JSG1178 without drug is comparable, while the $FliC^{-}$ $FljB^{+}$ JSG1179 strain was hindered in biofilm formation by an average of 45% compared to background JSG210 (data not shown). Since the loss of FljB did not produce a biofilm-deficient strain, and JSG1179 was attenuated in biofilm formation, one possible hypothesis is that both FliC and FljB play a positive role in biofilm formation, but FliC to a greater extent. The JSG1178 $FljB^{-}$ $FliC^{+}$ strain maintains effective biofilm formation because of its FliC expression. If T315 is binding directly to the FljB filament in *S.* Typhimurium to hinder biofilm formation processes, this may be occurring intracellularly, limiting FljB export and flagellar synthesis, at the cellular surface to coat existing FljB flagella, or both. None of the remaining above-mentioned *S.* Typhimurium surface appendage mutants experienced a significant difference in T315-induced anti-biofilm activity compared to appropriate background strains. These studies may be expanded by evaluating efficiency of T315 activity towards additional *S.* Typhimurium mutants, such as those with altered lipopolysaccharide expression, and polysaccharide/protein secretion profiles. It is also important to note that the compound

may be interfering with biofilm formation indirectly, such as by affecting signaling or quorum sensing pathways necessary for the initiation of the biofilm lifestyle.

4.3 Evaluation of the mechanism of action of anti-biofilm compound T315

Gaining understanding of the mechanism of action of T315 has been attempted via multiple approaches and is an ongoing effort. First, generation of a T315-resistant *S.* Typhimurium isolate was attempted with the goal of performing whole-genome sequencing on this isolate to identify genetic loci altered from the wild-type which allow the bacterium to persist in biofilm formation in the presence of T315. Exposure of biofilm-residing *S.* Typhimurium to repeated and stepwise increasing doses of T315 failed to generate an isolate with lasting resistance to the anti-biofilm agent. Upon increasing T315 dosage from 5 to 7.5 μM, initially observed "resistant" mutants did not retain this resistance. The idea of performing this process with a mutagenic *S.* Typhimurium strain to increase probability of a desirable mutation was considered, but ultimately not performed due to the challenges associated with evaluating individual contributions of a high number of genetic mutations to the T315-resistant phenotype within an obtained isolate.

Our ongoing approach to determine the mechanism of action of T315 is a direct pull-down of biotinylated T315 with its *S.* Typhimurium cellular targets. A polyethylene glycol (PEG) linker has been added to T315 at the site of the piperazine functional group via click chemistry, but analysis of this molecule indicates that the PEG addition at this site is detrimental to T315's functionality. Our collaborators in the lab of Jim Fuchs are currently working to synthesize T315 with a PEG addition at the site of the amide (T315-

PEG-ii). Once this molecule is synthesized, it will be evaluated to confirm that the PEG addition does not interfere with anti-biofilm activity of T315. Following this confirmation, a biotin marker will be added onto the PEG linker portion of T315-PEG-ii, and biotinylated T315 will be exposed to *S.* Typhimurium cellular lysate before utilizing biotin-streptavidin interactions to pull down T315 binding partners. Binding partners will be identified via mass spectrometry.

4.4 Conclusions and Future Directions

As a human-restricted pathogen, the carrier state of *S.* Typhi infection is fundamental to the spread of bacteria to new hosts and of consequential disease. The biofilm lifestyle within the gall bladder of carriers is an important aspect of chronic carriage that contributes to difficulties eradicating the spread of *Salmonella*. Used in conjunction with antibiotic therapy, anti-biofilm agents could be a useful therapeutic tool to limit chronic carriage and thus new *S.* Typhi infections.

The stability of T315 and Cp315 within solution has been a limitation of this study. The compounds should be stored dry and not exposed to light for long-term storage. Stock solutions should be prepared in dimethyl sulfoxide (DMSO) and stored at -20°C, as the compounds are most soluble in DMSO. However, concentrations of DMSO greater than 1% in solution is toxic to *Salmonella*, so these compounds must be diluted in 1x PBS directly before being administered. We believe this step of transfer into 1x PBS is the source of solubility issues and high variation of compound effectiveness between assays. Especially at concentrations over 10 μM, T315 is prone to precipitating out of

solution, which limited our EC_{50} studies to a narrow concentration range, as previously described.

T315 is currently being derivatized to produce potential anti-biofilm compounds with a lower EC_{50} and/or greater solubility in 1x PBS. A scheme for this derivitization is depicted in Figure 10. Once synthesized, these compounds will be screened for biofilm inhibition against *S.* Typhimurium 14028s. Studies towards the discovery of the mechanism of action of T315 will be continued once the synthesis of T315-PEG-ii has been achieved. Once synthesized, T315-PEG-ii will be evaluated for preservation of anti-biofilm activity to ensure that drug interactions have not been compromised by the addition of the polyethylene glycol linker. T315-PEG-ii will then be biotinylated, and this biotinylated compound will be utilized for direct pull down of T315 targets, which will be identified by mass spectrometry. Understanding the mechanism of action of T315 may provide novel insight into the *Salmonella* biofilm formation processes as well as direct future methods of synthesis of anti-biofilm agents to achieve diminution of the typhoid carrier state.

Tables

Strain	Characteristics	Reference
JSG210	*S.* Typhimurium ATCC 14028s (CDC6516-60); wild type	ATCC
JSG1547	JSG210 *motA595*::Tn10	Gift of T. Lino
JSG1169	*S.* Typhimurium SR11x4252	Gift of A. Baumler
JSG1174	JSG1169 Δ[*fim-aph*-11::Tn10]-*391 lpfC*::Kan *pefC*::Tet *agfB*::Cam	Gift of A. Baumler
JSG1178	JSG210 BC117 *hin*108::Tn10dCam (FljB off)	Gift of B. Cookson
JSG1179	JSG210 BC119 *fliC*::Tn10 *hin*108::Tn10dCam (FljB on)	Gift of B. Cookson
JSG3736	JSG210 Δ*csgA* via Wanner	Adcox, *et al.* [13]
JSG3828	*A. baumannii* 19606	Gift of D. Wozniak
JSG3906	*P. aeruginosa* PAO1	Gift of D. Wozniak

Table 1. Bacterial strains and relevant characteristics.

Figures

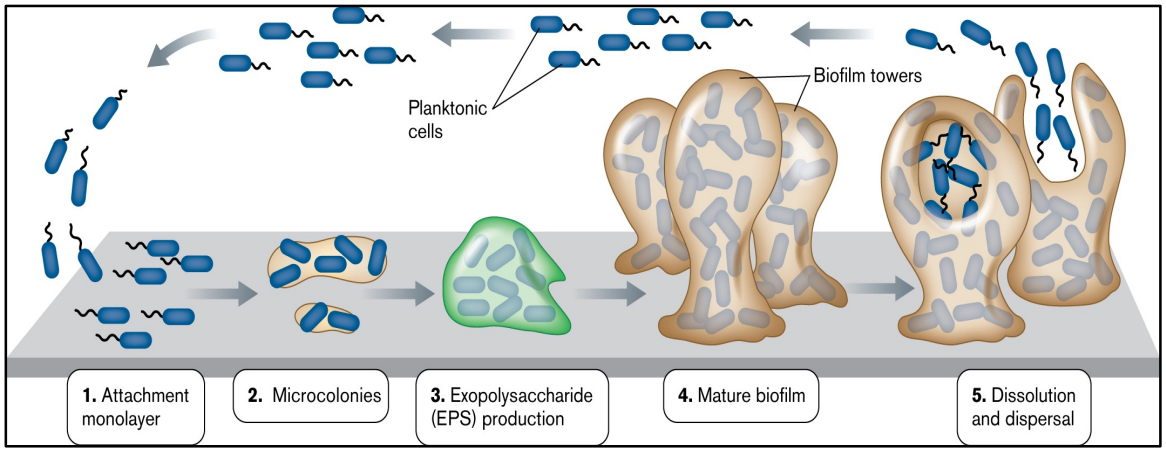


Figure 1. Phases of biofilm development. "Biofilms: Multicellular Microbes?" [14]. The biofilm lifecycle is initiated via attachment of planktonic microorganisms to a surface in formation of a monolayer. The formation of microcolonies correlates with changes in gene expression to transition to biofilm lifecycle, such as down-regulation of motility genes, and precedes secretion of exopolysaccharide/extrapolymeric substance (EPS). Mature biofilms form characteristic biofilm towers encased in EPS, and are recalcitrant to immune and antimicrobial clearance. Return of biofilm members to planktonic state may be facilitated by passive detachment or active dispersal.

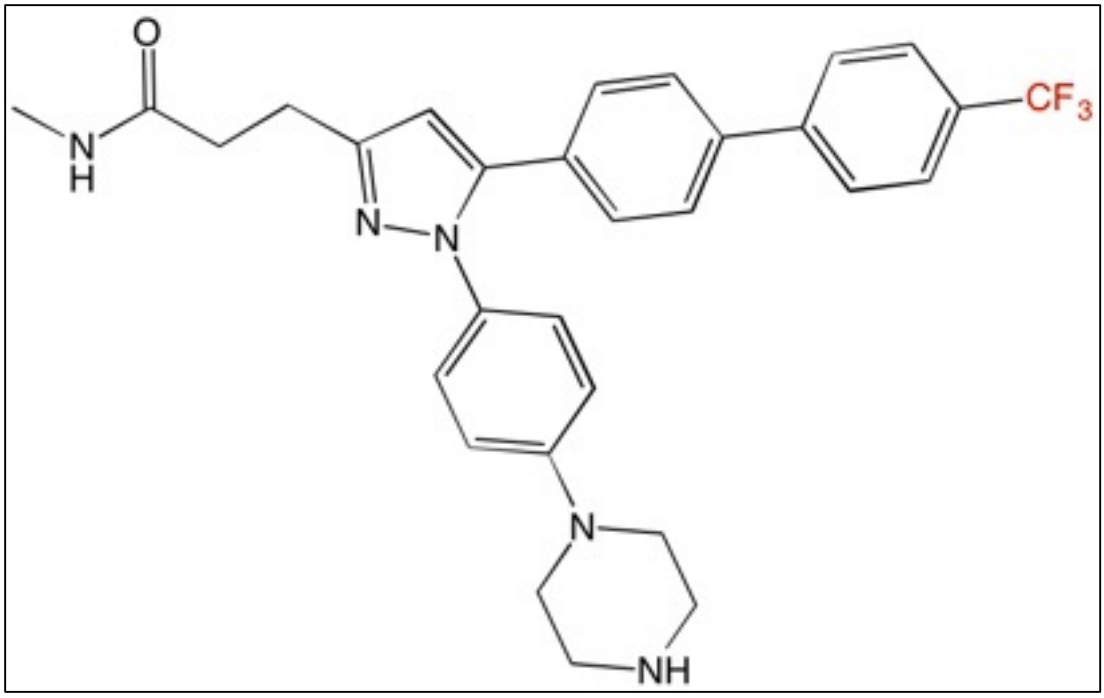


Figure 2. Chemical structure of T315. Regions of structural similarity between T315 and Cp315 are depicted in black while regions of dissimilarity between the compounds are depicted in red.

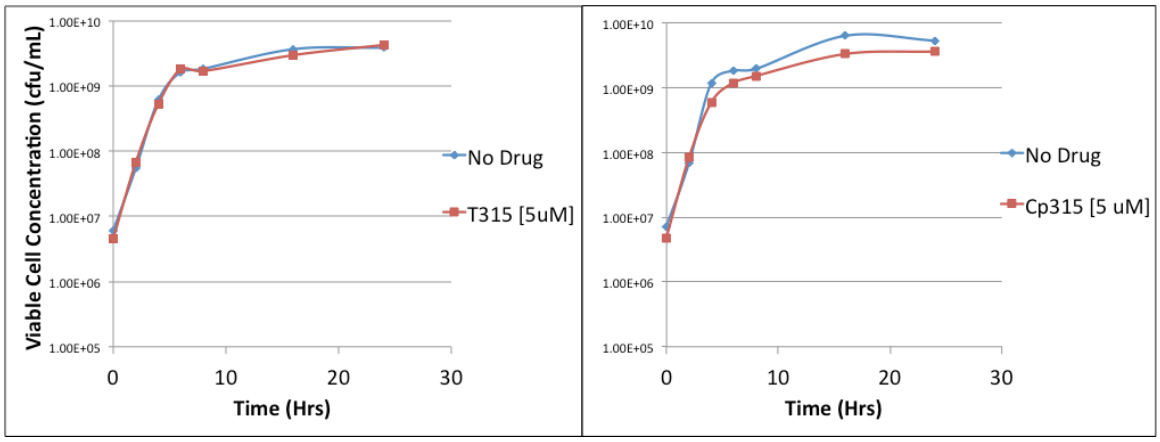
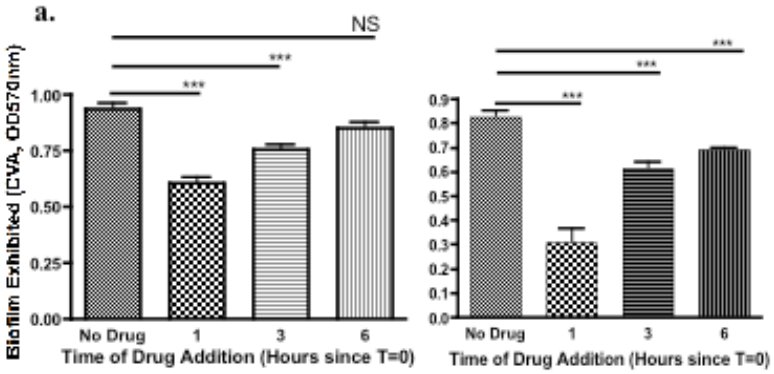


Figure 3. Viability assays of *S.* Typhimurium in presence of 5 µM T315 and Cp315. Results indicate that reduction of biofilm observed in Rapid Attachment Assays was not due to bacteriostatic or bactericidal effects of T315 or Cp315, as the growth curves observed in presence of each drug are not markedly different from those without drug. This experiment was performed once.

a.



b.

% Inhibition of Biofilm		
Time Drug Added (h)	Drug Added [5 uM]	
	T315	Cp315
1	41.5% ***	48.5% **
3	30.1% ***	25.3% ***
6	19.5% *	17.7% ***

Figure 4. (a) **Effects of delayed drug delivery on biofilm formation.** Biofilms were visualized via crystal violet assay 24h post final drug addition (5 μM). T315 on left panel, Cp315 on right panel. Early Addition Dispersion Assays utilized a similar methodology to Rapid Attachment Assays, but delayed delivery of drug to T=1, 3, and 6h post initial incubation. Results indicate that delivery of drug at T=1 and T=3 significantly decrease biofilm formation in both drugs, and delivery at T=6 significantly decreases biofilm formation in Cp315 only. Shown are results from one assay performed in 8 technical replicates. ***$p<0.001$ (b) Average percent inhibition of biofilm observed under delayed drug delivery using results from three biological replicates. ***$p<0.001$, **$p<0.01$, *$p<0.05$

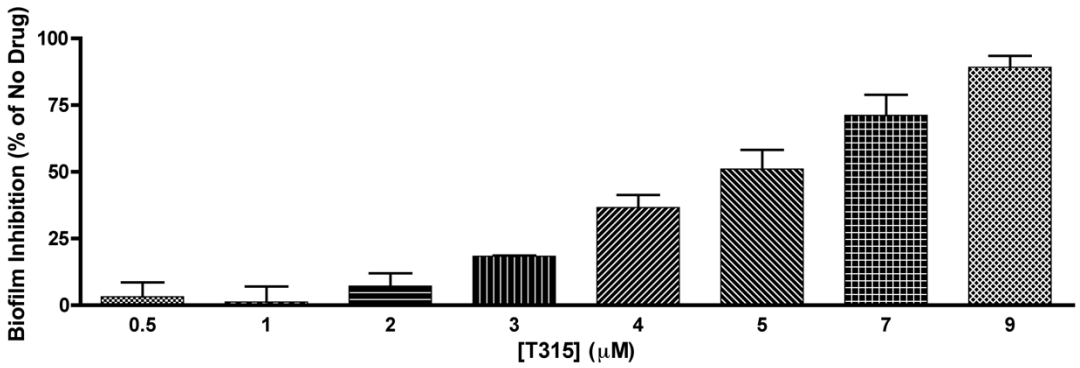


Figure 5. Half maximal effective concentration (EC_{50}) determination of T315. Determination of the half-maximal effective concentration of T315 was achieved by exposing JSG210 to drug concentrations from 0.5 to 9 μM and examining the levels of subsequent anti-biofilm activity after 24h via crystal violet staining technique. The EC_{50} was determined to be 4.61 μM with a 95% confidence interval from 3.13 to 6.78 μM.

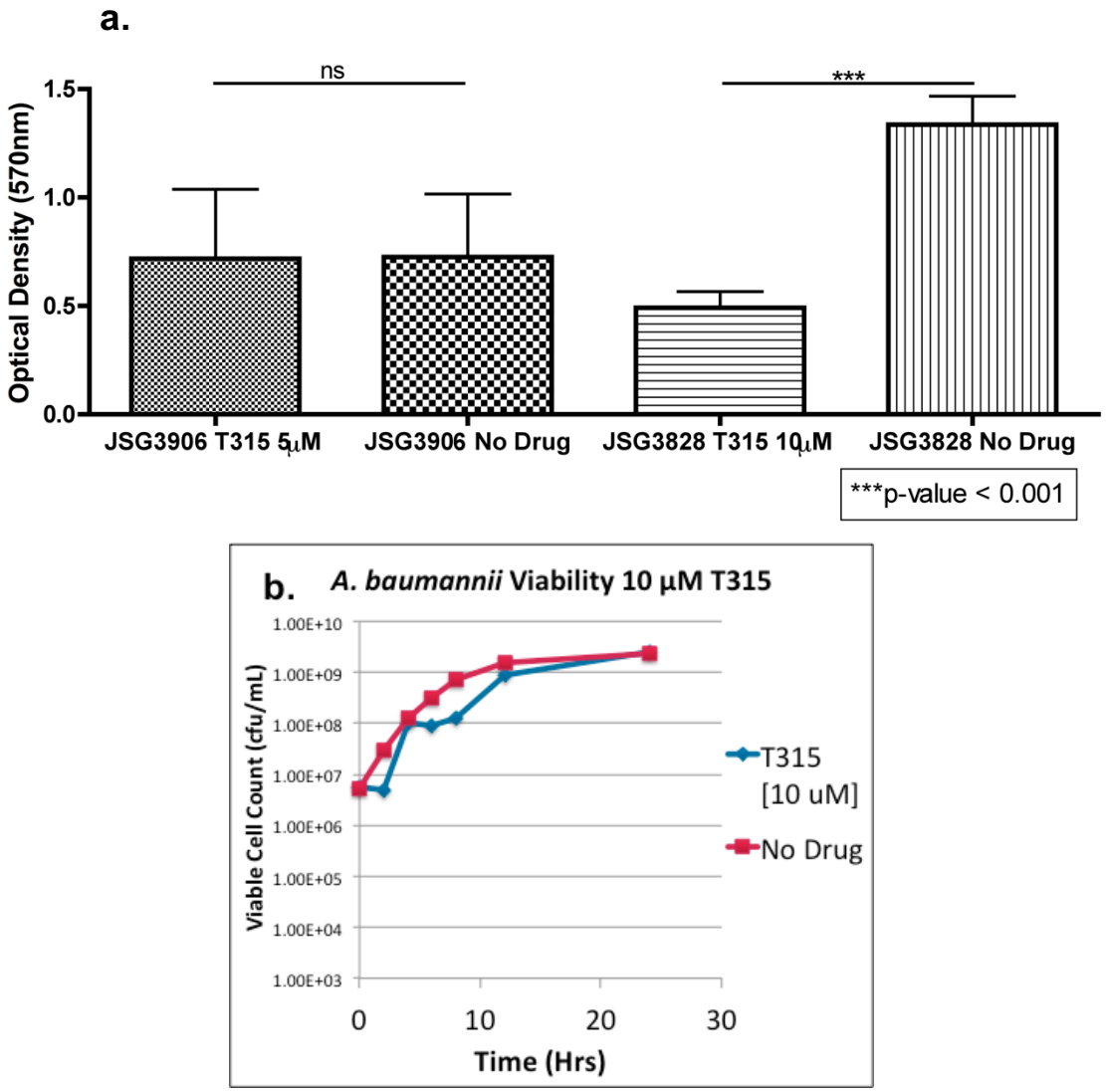


Figure 6. Evaluation of T315 anti-biofilm effects on *P. aeruginosa* (JSG3906) and *A. baumannii* (JSG3828). (a) After 24h exposure of 10 μM T315, JSG3828 biofilm formation was reduced by an average of 61.2%, quantified via crystal violet assay. Exposure of JSG3906 to 5 μM T315 did not significantly affect biofilm formation after 24h. ***p<0.001. (b) Enumeration of viable JSG3828 at timepoints over 24h exposure to 10 μM T315 suggests that anti-biofilm activity is not a resultant of bacteriostatic or bactericidal effects. This assay was performed once.

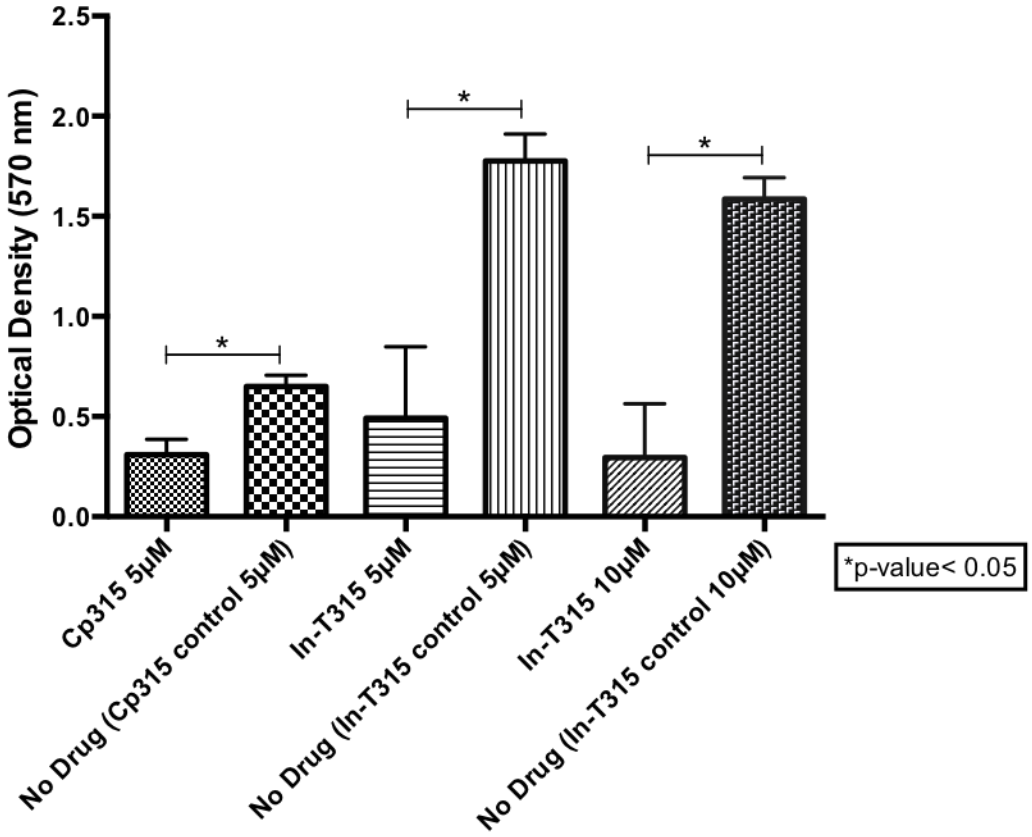


Figure 7. T315-related compounds Cp315 and In-T315 effects on *S.* Typhimurium biofilm formation. 24h crystal violet assays indicate that both Cp315 and In-T315 significantly inhibit biofilm formation of JSG210 at 5 μM, by 58.4% and 78.4%, respectively. In-T315 was seen to inhibit biofilm formation at 10 μM.by 83.3%.

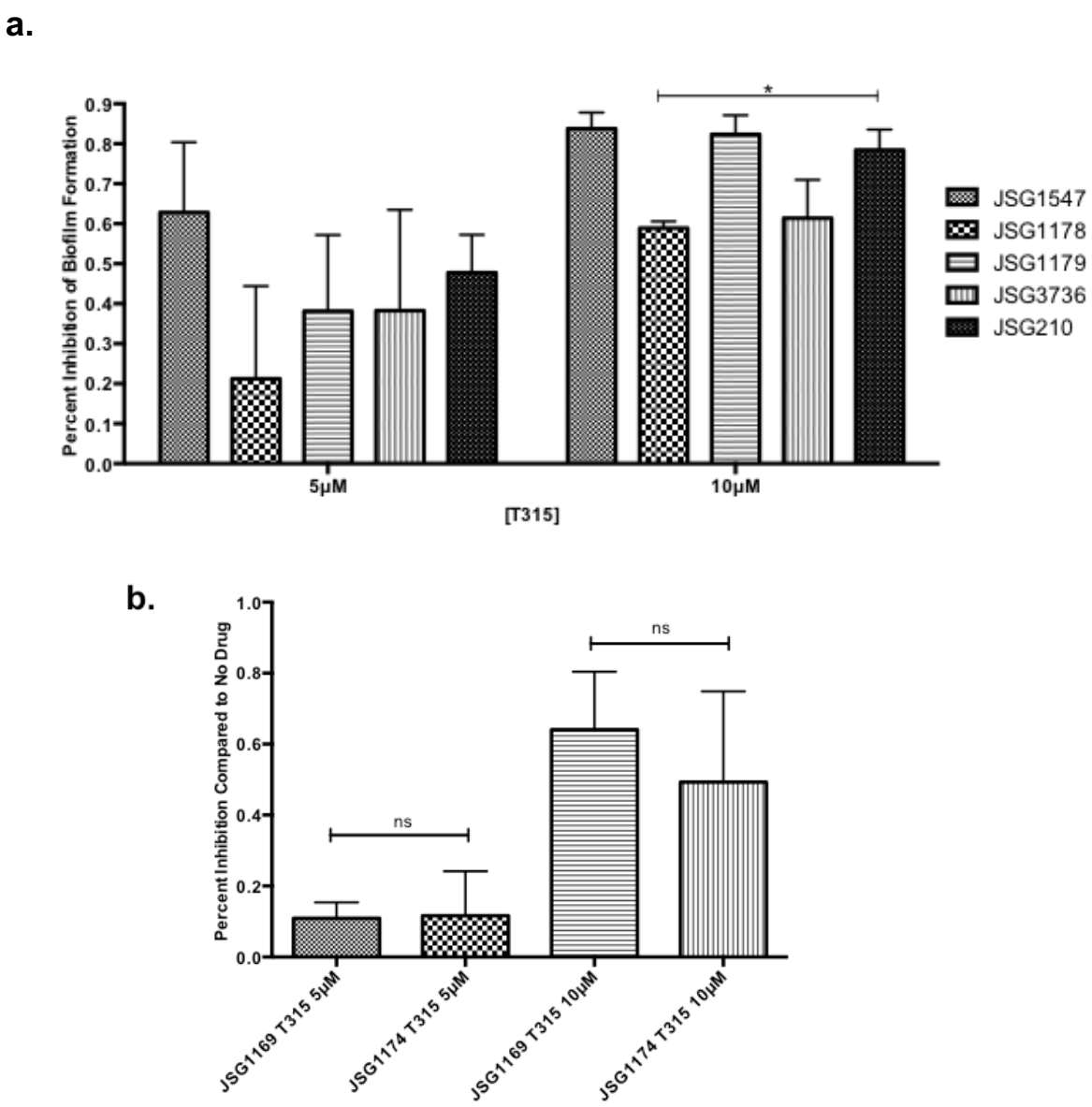


Figure 8. Evaluation of T315 anti-biofilm activity against S. Typhimurium flagella curli fimbriae, and motility mutants. Biofilm formation of each bacterial strain with and without the presence of 5 and 10 μM T315 was quantified after 24h by crystal violet staining. These data were used to compute percent inhibition of biofilm formation caused by the compound, shown above. (a) JSG1178 was seen to experience an average percent inhibition of biofilm formation significantly different than the wild type JSG210 background strain when exposed to 10 μM T315. In these studies, JSG1178 exposed to 10 μM T315 exhibited 58.9% inhibition of biofilm formation while JSG210 exhibited 78.4%. $p<0.05$. (b) No significant difference between T315-induced biofilm inhibition was observed between JSG1174 fimbriae mutant and JSG1169 SR11 wild type background.

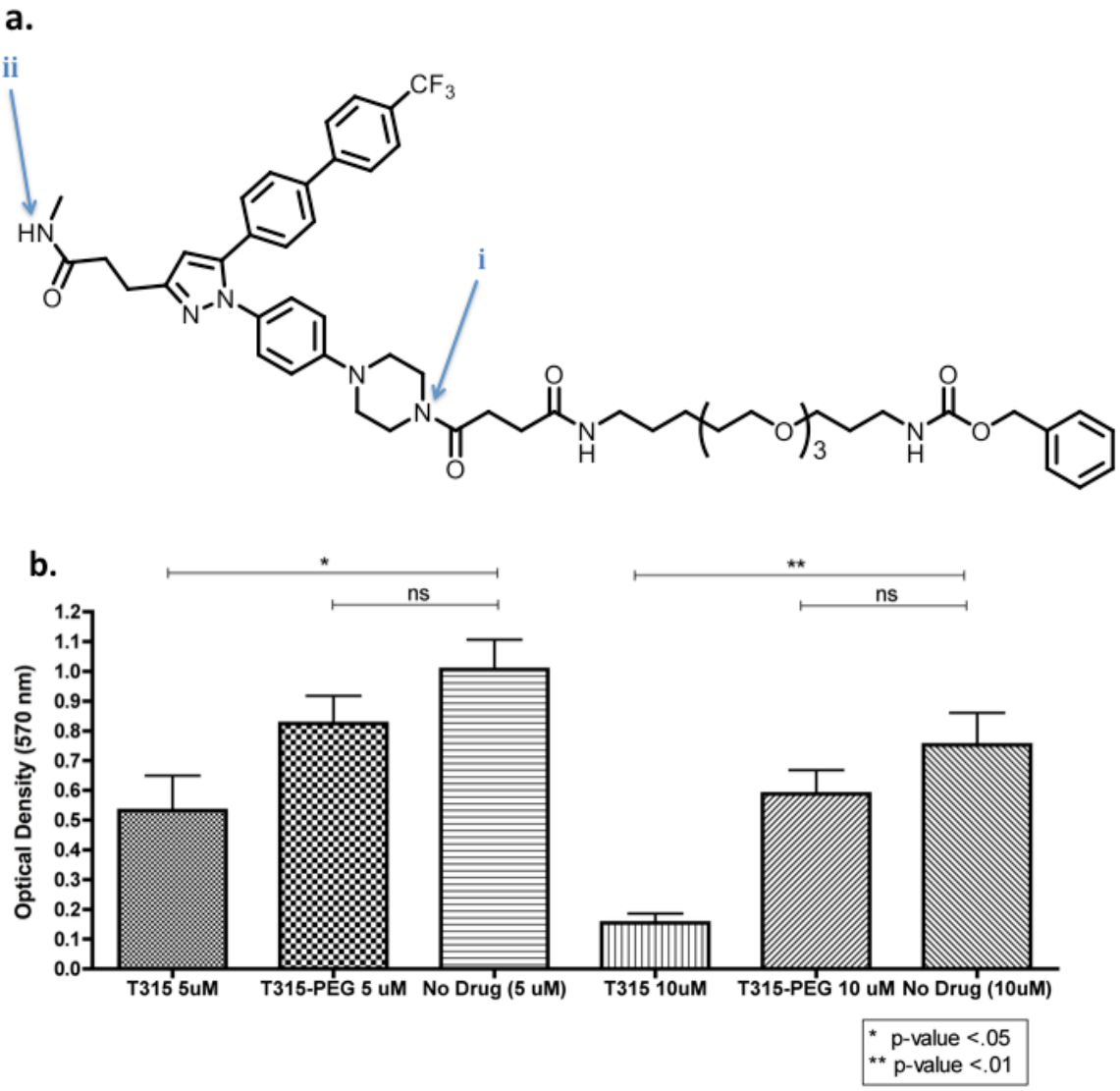


Figure 9. (a) Structure of T315-PEG-i. Sites of polyethylene glycol linker attachment for T315-PEG-i and T315-PEG-ii are indicated in blue. (i) pyrazole, (ii) amide site. **(b)** Loss of biological activity of T315-PEG-i. 24h drug exposure followed by crystal violet staining indicates that T315-PEG-i does not significantly reduce biofilm formation of *S.* Typhimurium at 5 or 10 μM concentrations.

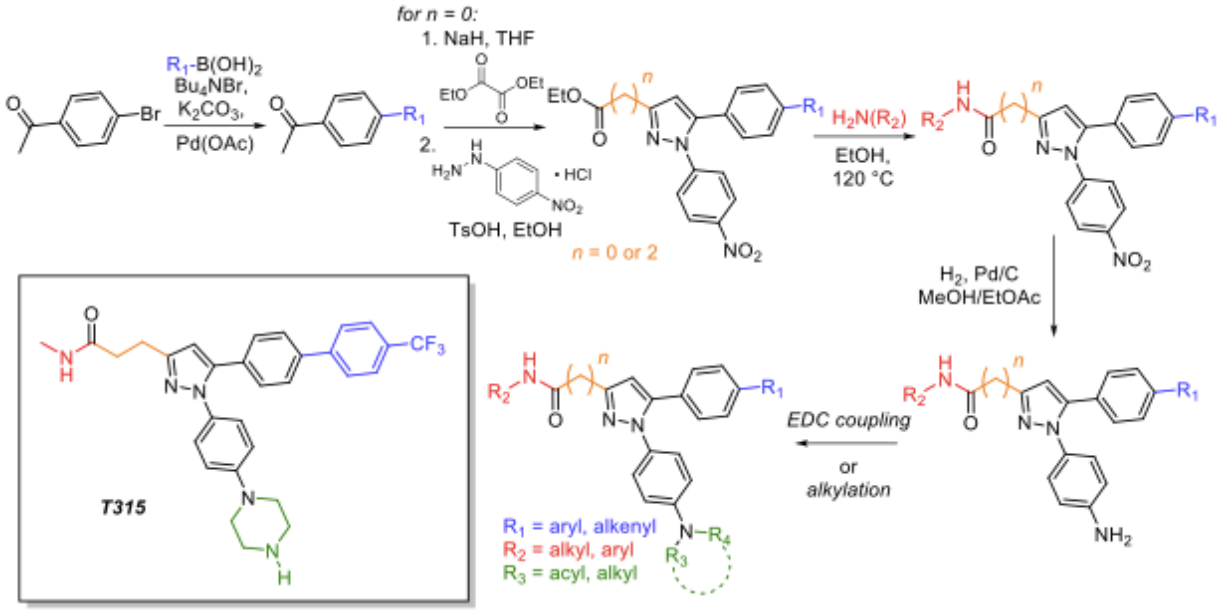


Figure 10. Scheme for the derivatization of key functional moieties of T315 based on the established synthesis of T315 and related analogues for cancer screening.

References

1. LaRock, D.L., A. Chaudhary, and S.I. Miller, *Salmonellae interactions with host processes.* Nat Rev Microbiol, 2015. **13**(4): p. 191-205.
2. Havelaar, A.H., et al., *World Health Organization Global Estimates and Regional Comparisons of the Burden of Foodborne Disease in 2010.* PLoS Med, 2015. **12**(12): p. e1001923.
3. Crump, J., S. Luby, and E. Mintz, *The global burden of typhoid fever.* Bull World Health Organ, 2004. **82**(5): p. 346-353.
4. Parry, C.M., et al., *Typhoid fever.* N Engl J Med, 2002. **347**(22): p. 1770-82.
5. Gonzalez-Escobedo, G., J.M. Marshall, and J.S. Gunn, *Chronic and acute infection of the gall bladder by Salmonella Typhi: understanding the carrier state.* Nature Reviews Microbiology, 2011. **9**(1): p. 6.
6. Merselis, J.G., Jr., et al., *Quantitative bacteriology of the typhoid carrier state.* Am J Trop Med Hyg, 1964. **13**: p. 425-9.
7. Crawford, R.W., et al., *Gallstones play a significant role in Salmonella spp. gallbladder colonization and carriage* Proceedings of the National Academy of Sciences, 2010. **107**(9): p. 4353-4358.
8. Costerton, J.W., et al., *Microbial biofilms.* Annu Rev Microbiol, 1995. **49**: p. 711-45.
9. Liu, T.M., et al., *OSU-T315: a novel targeted therapeutic that antagonizes AKT membrane localization and activation of chronic lymphocytic leukemia cells.* Blood, 2014.

10. Lee, S.L., et al., *Identification and characterization of a novel integrin-linked kinase inhibitor.* J Med Chem, 2011. **54**(18): p. 6364-74.

11. Crawford, R.W., K.E. Reeve, and J.S. Gunn, *Flagellated but not hyperfimbriated Salmonella enterica serovar Typhimurium attaches to and forms biofilms on cholesterol-coated surfaces.* J Bacteriol, 2010. **192**(12): p. 2981-90.

12. Teplitski, M., A. Al-Agely, and B.M. Ahmer, *Contribution of the SirA regulon to biofilm formation in Salmonella enterica serovar Typhimurium.* Microbiology, 2006. **152**(Pt 11): p. 3411-24.

13. Adcox, H.E., et al., *Salmonella extracellular matrix components influence biofilm formation and gallbladder colonization.* In preparation.

14. Slonczewski, J., Foster, JW., Gillan KM, *Microbiology: An Evolving Science*. 3rd ed. 2014, London: W.W. Norton.